

Prevalent genotypes of meticillin-resistant *Staphylococcus aureus*: report from Pakistan

A. Zafar,¹ M. Stone,² S. Ibrahim,¹ Z. Parveen,¹ Z. Hasan,¹ E. Khan,¹ R. Hasan,¹ J. Wain³ and K. Bamford²

Correspondence

A. Zafar
afia.zafar@aku.edu

¹Aga Khan University Hospital, Karachi, Pakistan

²Department of Microbiology, Imperial College Healthcare NHS Trust, London, UK

³Laboratory of Enteric Pathogens, Health Protection Agency, London, UK

Meticillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen in Pakistan and is emerging in the community. This is one of the first reports of the prevalent genotypes of MRSA in both hospital and community settings in Pakistan. Isolates collected in 2006–2007 were characterized by PFGE, staphylococcal cassette chromosome *mec* (SCC*mec*) typing and multilocus sequence typing (MLST). PFGE identified nine pulsotypes, the majority of isolates belonging to pulsotypes A ($n=70$) and B ($n=38$), which were predominant among hospital-onset MRSA (HO-MRSA) and community-onset MRSA (CO-MRSA) isolates, respectively. Among the HO-MRSA isolates, variants of SCC*mec* type III were prevalent, whilst SCC*mec* type IV or variants were predominant in the CO-MRSA isolates. MLST identified two principal sequence types, ST8 and ST239. An association was observed between ST8, PFGE pulsotype B and SCC*mec* type IV in the CO-MRSA (ST8-MRSA-IV). Similarly, ST239, PFGE pulsotype A and SCC*mec* type III were associated with HO-MRSA (ST239-MRSA-III). Therefore, the prevalent genotypes circulating in Pakistan at the time of study were ST8-MRSA-IV and ST239-MRSA-III in the community and hospital settings, respectively. A set of HO-MRSA isolates collected in 1997 were characterized by PFGE and SCC*mec* typing for comparison. The isolates belonged to two PFGE pulsotypes (A, $n=28$; B, $n=11$) and contained just two SCC*mec* types. These results suggest that an increase in genetic diversity occurred over the period 1997–2007 as a result of either microevolution or the importation of strains from surrounding areas.

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INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA) is an important global pathogen in the hospital setting that has become increasingly prevalent in community-acquired infections in recent years (Fluit *et al.*, 2001; Chambers, 2001). There are limited data regarding the frequency of healthcare-associated infections caused by MRSA in Pakistan, but recent studies suggest that 42–51% of healthcare-associated *S. aureus* infections may be due to MRSA (Hafiz *et al.*, 2002; Butt *et al.*, 2004; Perwaiz *et al.*, 2007).

Clinical *S. aureus* isolates have traditionally been tracked using phage typing and PFGE, which have proved useful in local outbreak investigations, but are less suitable for global studies (Bannerman *et al.*, 1995; Branger *et al.*, 2003;

Ichiyama *et al.*, 1991; Struelens *et al.*, 1992). DNA-sequence-based methods including multilocus sequence typing (MLST) (Maiden *et al.*, 1998), *spa* typing (Shopsin *et al.*, 1999) and *coa* typing (Shopsin *et al.*, 2000) have demonstrated adequacy for use in global epidemiological studies and for determining genetic relationships. Sequence-based methods are commonly supplemented by the use of staphylococcal cassette chromosome *mec* (SCC*mec*) typing of MRSA (Chongtrakool *et al.*, 2006).

Using MLST, *S. aureus* populations have been shown to be highly clonal, with the majority of isolates belonging to a limited number of closely related genotypes, which are often globally distributed and stable over time (Feil & Enright, 2004; Enright *et al.*, 2002). Studies have shown that epidemic MRSA (EMRSA) clones from hospitals belong to one of five clonal complexes: CC5 (New York/Japanese clone, Paediatric clone), CC8 (Iberian clone, Brazilian clone, Archaic clone), CC22 (EMRSA-15), CC30 (EMRSA-16) and CC45 (Berlin epidemic clone) (Enright *et al.*, 2002; Aires de Sousa *et al.*, 2000; Sá-Leão *et al.*, 1999;

Abbreviations: CO-MRSA, community-onset MRSA; HO-MRSA, hospital-onset MRSA; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

Dominguez *et al.*, 1994; Heym *et al.*, 2002; Teixeira *et al.*, 1995; Crisóstomo *et al.*, 2001; Johnson *et al.*, 2001; Witte *et al.*, 1994). The aim of this study was to determine the prevalent MRSA genotypes circulating in the Aga Khan University Hospital (AKUH) in Karachi and the surrounding areas between June 2006 and July 2007 using PFGE, MLST, SCCmec typing and antimicrobial susceptibility testing.

METHODS

Isolates. A total of 126 consecutive, non-duplicated, clinically significant MRSA isolates were collected at the AKUH, Karachi, between June 2006 and July 2007. Of the 126 isolates, 102 were from Karachi, 12 from Sindh, 8 from Punjab and 4 from the North-West Frontier Province. To compare genotypes over time, 39 isolates collected from patients admitted to AKUH in 1997 were also investigated.

MRSA isolates from samples taken ≥ 48 h after hospital admission were considered to be hospital-onset (HO-MRSA, $n=107$). The 19 remaining isolates were from samples taken within 48 h of admission from patients with no previous history of hospital admission in the last 12 months. However, as it was not possible to exclude all potential risk factors, the latter isolates were referred to as community-onset (CO-MRSA) rather than community-acquired.

Bacterial identification and antimicrobial susceptibility testing.

S. aureus was identified using coagulase, DNase and mannitol fermentation tests. Antimicrobial susceptibility was determined using Kirby–Bauer disc diffusion in accordance with the Clinical and Laboratory Standards Institute guidelines using oxacillin (1 µg) or cefoxitin (30 µg), erythromycin (15 µg), gentamicin (10 µg), amikacin (30 µg), co-trimoxazole (1.25/23.75 µg), chloramphenicol (30 µg), ofloxacin (5 µg), tetracycline (30 µg) and vancomycin (30 µg) (NCCLS, 2004). Susceptibility to fusidic acid (10 µg) was determined according to the British Society of Antimicrobial Chemotherapy guidelines (BSAC, 2008).

DNA extraction. DNA was extracted from *S. aureus* isolates using the Wizard Genomic DNA Purification kit according to the manufacturer's instructions (Promega).

mecA PCR. The presence of the *mecA* gene was confirmed by PCR amplification as described by Murakami *et al.* (1991).

PFGE. PFGE was performed as described by McDougal *et al.* (2003). Briefly, plugs were digested with *Sma*I restriction enzyme (Promega) and loaded into the wells of a 1% agarose gel and run in $0.5 \times$ TBE using a CHEF-DR III system (Bio-Rad) according to the following parameters: 200 V (6 V cm^{-1}), temperature 14°C , initial switch time 5 s, final switch time 40 s, included angle 120° , with a total run time of 21 h.

Visual analysis of PFGE fingerprints was performed according to the criteria of Tenover *et al.* (1995). Similarity between banding patterns was estimated using BioNumerics software (version 4.5; Applied Maths) by performing cluster analysis using the unweighted pair group method analysis (UPGMA). Isolates that showed a coefficient of similarity $\geq 75\%$ (a difference of four bands) were grouped as one PFGE type and labelled alphabetically. Similarly, those showing a coefficient of similarity of $\geq 50\%$ (a difference of \geq seven bands) were grouped as a separate PFGE type. Isolates that showed between 50% and 75% similarity (a difference of five or six bands) were regarded as PFGE subtypes.

SCCmec typing. SCCmec typing was performed according to the multiplex PCR protocol of Oliveira & de Lencastre (2002). *S. aureus* strains COL, BK2464, ANS46 and MW2 were used as controls for SCCmec types I, II, III and IV, respectively. In instances where the SCCmec type could not be determined, the *mec* and *ccr* gene complexes were typed according to Zhang *et al.* (2005) and Hanssen *et al.* (2004), respectively.

MLST. MLST was performed according to the protocol of Enright *et al.* (2000).

Statistical analysis. SPSS version 16.0 was used for data analysis. Differences in the antimicrobial susceptibility of HO-MRSA and CO-MRSA isolates to the agents were compared using the chi-square test. A *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

PFGE

Two PFGE pulsotypes (A, $n=28$; B, $n=11$) with eight subtypes were observed among the 39 isolates collected in 1997, in five major clusters of A1, A4, A16, A17 and B2.

The PFGE patterns of the 126 MRSA isolates collected in 2006–2007 were classified into nine pulsotypes with 33 subtypes. The predominant pulsotypes were A ($n=22$) and B ($n=11$), with multiple subtypes, A1–A15 ($n=48$) and B1–B10 ($n=27$). These two pulsotypes accounted for 108 (86%) isolates. Other pulsotypes, C, D, E and F, were less common with fewer subtypes, C1 and C2, D1, E1–E4 and F1, respectively. Pulsotypes G–I had no subtypes.

Interestingly, subtypes A16 and A17 observed in 1997 were not observed in the 2006–2007 collection, indicating a change in the prevalence of molecular subtypes. This finding was in agreement with other studies published from Poland, Taiwan, Portugal and Spain (Tai *et al.*, 2006; Huang *et al.*, 2006; Bogut *et al.*, 2008; Łuczak-Kadłubowska *et al.*, 2008; Aires de Sousa *et al.*, 2008). Furthermore, persistence and overall predominance of PFGE pulsotypes A and B during both periods indicated that these were the local epidemic strains. These findings suggested their distinct origin and were in full agreement with reports published from neighbouring countries, including India, China and Taiwan (Ko *et al.*, 2005; Shukla *et al.*, 2004; Vandenesch *et al.*, 2003).

CO-MRSA ($n=19$) was distributed in five PFGE pulsotypes (A, B, E, H and I), whilst the HO-MRSA ($n=107$) was distributed in six PFGE pulsotypes (A, B, C, D, F and G). These results indicated the predominance and spread of PFGE pulsotype A in the hospital setting during both study periods, which was consistent with previous reports (Wang *et al.*, 2002; Tai *et al.*, 2006). The results also indicated that the same genetic lineages of *S. aureus* had disseminated and persisted within the hospital setting and were also spreading in the community. The dispersion of CO-MRSA in multiple PFGE pulsotypes suggested their diverse origin as a result of either microevolution or the importation of strains from surrounding areas, some of which had become established in

the hospital setting. Nosocomial transmission of CO-MRSA and hospital outbreaks have been described in the USA and Europe (Otter & French, 2006).

SCCmec typing

SCCmec typing of the 2006–2007 isolates identified eight SCCmec types circulating in Pakistan (Table 1). This analysis indicated that among the 126 isolates studied, 78 contained a SCCmec type III or type III variant and 38 contained a type IV or type IV variant. Nine isolates could not be assigned to a SCCmec type due to the lack of amplification, but were subsequently found to belong to SCCmec type IV following typing of the *mec* and *ccr* gene complexes (*mec* gene complex B, *ccr* gene complex 2) and were tentatively named SCCmec type IV variant 1. A further isolate contained a unique type that was identified as SCCmec type IVA lacking a 342 bp band and was named SCCmec type IV variant 2. The distribution of the various PFGE pulsotypes in the CO-MRSA and HO-MRSA isolates and their relation with SCCmec type are shown in Table 1.

In the hospital setting, the predominant element was SCCmec type III variant 1, and in the community, the most frequent types were IVA and IV. SCCmec type IVA was found to be distributed most widely, being detected in seven of the nine PFGE pulsotypes, while SCCmec type IV was distributed in three PFGE pulsotypes (Table 1). Similarly, SCCmec type IV variant 2 was found in three different PFGE pulsotypes. These strains were isolated from different geographical regions and displayed no epidemiological linkage. This finding is similar to that of Berglund *et al.* (2005), who reported that the SCCmec type IV element was widely distributed in PFGE pulsotypes in Sweden, suggesting that the SCCmec type IV element was linked to a capacity to transfer between distinct clones of *S. aureus*. HO-MRSA collected during 1997 was of only two types, SCCmec type III variant 1 and IVA, while the 2006–2007 isolates demonstrated greater diversity. The results of SCCmec typing were consistent with studies from various

Asian countries where SCCmec type III elements predominated, while in the USA, Japan and Korea, the most prevalent SCCmec types were I and II (Chongtrakool *et al.*, 2006; Ito *et al.*, 2004; Ko *et al.*, 2005; Ma *et al.*, 2002; Shukla *et al.*, 2004; Vandenesch *et al.*, 2003). Recently, Chongtrakool *et al.* (2006) reported that isolates containing SCCmec type III were prevalent in 9 out of 11 Asian countries. These findings illustrated the evolution of organisms under variable geographical and environmental pressures. Further understanding of endemic MRSA lineages and their correlation with regional and global strains requires further investigation.

MLST

Eighteen of the CO-MRSA isolates and representatives of the major HO-MRSA PFGE pulsotypes/subtypes ($n=25$) were further characterized by MLST. Among the CO-MRSA isolates, there was a significant association with ST8 (chi-square test, Yates' correction, $P \leq 0.05$), which was the prevalent sequence type ($n=12$; 67%). In addition, ST239 was represented by three isolates and the remaining sequence types were each represented by one isolate. Among the 25 HO-MRSA isolates, only two sequence types were observed. ST239 was represented by 14 isolates and ST8 was represented by 11 isolates. No significant association was observed with a particular sequence type among the HO isolates (chi-square test, Yates' correction, $P \geq 0.05$). Robinson & Enright (2004) demonstrated that ST239 evolved from ST8 following a large-scale recombination event which led to the incorporation of ~557 kb (spanning the origin of replication, *oriC*) from a ST30 donor. ST239 has previously been shown to be prevalent in many other Asian countries, including China, India, Indonesia, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam and Hong Kong (Chongtrakool *et al.*, 2006; Zhang *et al.*, 2005). Shabir *et al.* (2010) recently reported the prevalence of ST239 among MRSA isolates collected from Pakistan and India. Interestingly, only three SCCmec

Table 1. Association of CO- and HO-MRSA with SCCmec type and PFGE pulsotypes

SCCmec type	PFGE pulsotypes (no. of isolates)		Total
	CO	HA	
III variant 1	A (3)	A (63), B (4), F (1)	71
IIIA*	—	A (3), D (1), F (1)	5
IIIB*	—	C (1)	1
III variant 2	B (1)	—	1
IV*	B (2), E (2), I (1)	B (2)	7
IVA*	B (5), E (2), H (1)	A (1), B (18), C (1), D (2), G (1)	31
IV variant 1	B (1), E (1)	B (4), C (3)	9
IV variant 2	—	B (1)	1
Total	19	107	126

*SCCmec variants previously described in the literature.

types were detected (III, IIIa and IV) in the isolates of their collection. In contrast, the majority of clinical MRSA isolates in Korea and Japan belong to ST5 (Ko *et al.*, 2005).

Five of the 23 isolates that belonged to ST8 contained a *SCCmec* type IV element (Fig. 1a). A further 14 isolates contained a *SCCmec* type IVA element, and three isolates contained the *SCCmec* type IV element (variant 1) that lacked the 342 bp band as described previously by Shore *et al.* (2005). A further isolate was found to contain a *SCCmec* type IVA variant (Ito *et al.*, 2003). These results suggested that ST8 had been introduced into the sample population on multiple occasions rather than a single introduction followed by subsequent diversification.

Of the 17 isolates designated ST239 by MLST, 15 contained *SCCmec* type III variant 1 (Fig. 1a). One isolate contained a *SCCmec* type IIIA element and the remaining isolate contained a *SCCmec* type IIIB element. Two of the three remaining isolates, which belonged to ST1 and ST217,

contained a *SCCmec* type IV element, whilst the final isolate, which belonged to ST1175, contained a *SCCmec* type IV variant 1 element. Therefore, the prevalent genotypes of MRSA found in Pakistan are ST239-MRSA-III and ST8-MRSA-IV.

Okuma *et al.* (2002) demonstrated a significant difference in the doubling time of ST239 compared to that of its parent sequence types (ST8 and ST30). The reasons for this were unclear, but may have been the result of the acquisition of a large amount of genetic material from an unrelated background, which may have been confounded by the presence of the large (~66 kb) *SCCmec* type III element, which encodes resistance to multiple antibiotics, antiseptics and heavy metals (Ito *et al.*, 2001). The presence of the *SCCmec* type III element may have conferred a selective advantage to ST239 in the hospital environment, despite the longer doubling time. In contrast, the CO-MRSA isolates in this study predominantly belonged to ST8, which, as already discussed, demonstrated a moderate

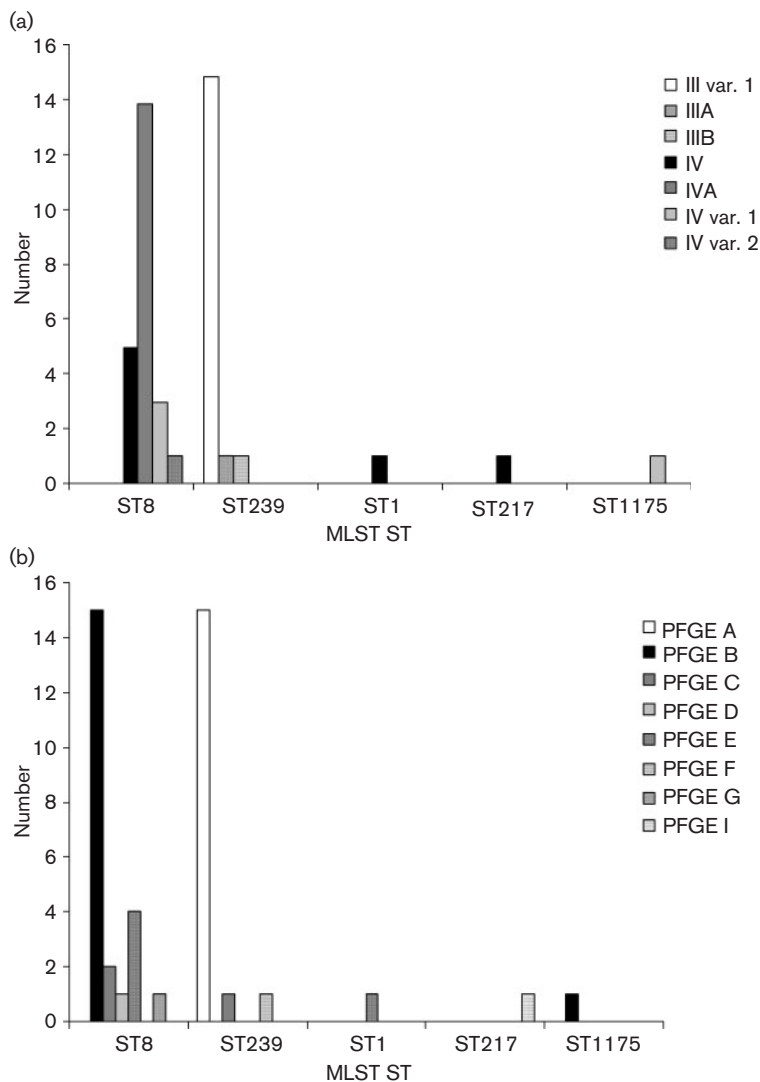


Fig. 1. Association of *SCCmec* type (a) and PFGE pulsotype (b) with MLST sequence type.

doubling time. MRSA isolates belonging to ST8 usually carry a type IV *SCCmec* element, which, at ~21 kb, is the smallest of the *SCCmec* elements described and encodes only resistance to methicillin (Ma *et al.*, 2002). Therefore, the small size of the *SCCmec* type IV element may not exert a large fitness cost on those strains carrying it, thus offering a selective advantage in the community setting.

MRSA isolates belonging to ST8 and ST239 displayed heterogeneity with regard to the PFGE pulsotypes (Fig. 1b). ST239 ($n=17$) was found within PFGE pulsotypes A ($n=15$), C ($n=1$) and F ($n=1$), whilst ST8 ($n=23$) was found within PFGE pulsotypes B ($n=15$), C ($n=2$), D ($n=1$), E ($n=4$) and G ($n=1$). These results suggested that ST8 and ST239 were introduced some time ago and had since diversified as demonstrated by the presence of multiple PFGE pulsotypes within ST8 and ST239. However, in light of the number of *SCCmec* variants observed, it is more probable that there had been multiple introductions followed by diversification.

Antimicrobial susceptibility

Overall, antimicrobial resistance of the 126 isolates was high: 96 % were resistant to gentamicin and ofloxacin, 84 % to erythromycin, 82 % to tetracycline and 79 % to amikacin. In addition, 43 % of isolates demonstrated resistance to co-trimoxazole, 18 % to fusidic acid and 10 % to chloramphenicol. All isolates demonstrated resistance to oxacillin and susceptibility to vancomycin. Resistance to ofloxacin was high in both HO-MRSA (97.2 %) and CO-MRSA (89.5 %) isolates. The only significant difference ($P<0.05$) between the HO-MRSA and CO-MRSA isolates was noted in susceptibility to gentamicin and tetracycline.

Previous studies have shown that the isolation of multi-antimicrobial-susceptible CO-MRSA isolates could predict the presence of *SCCmec* IV and IVA and this characteristic may be used as a surrogate marker for their recognition (Huang *et al.*, 2006; Pérez-Roth *et al.*, 2004; Takizawa *et al.*, 2005). However, this feature was not applicable to our study as the isolates were found to be additionally resistant to other antimicrobial agents. This may have reflected the practice of over-the-counter sale of antimicrobial agents leading to unrestricted and inappropriate use of these agents, which ultimately contributed to the emergence of resistance. This issue has been highlighted in various reports in recent years (Das *et al.*, 2001; Siddiqi *et al.*, 2002; Zafar *et al.*, 2008). However, as it was not possible to identify all antimicrobial exposures for the patients from whom the MRSA was isolated, this remains speculative.

It was observed that all PFGE pulsotype B CO-MRSA isolates (ST8-MRSA-IV) were resistant to co-trimoxazole, in comparison to only 10 % of PFGE pulsotype A isolates (ST239-MRSA-III), mostly comprising HA-MRSA ($P<0.001$). All ST8-MRSA-IV isolates were found to be susceptible to chloramphenicol. Susceptibility to amikacin was also noted in ST8-MRSA-IV isolates. In addition, all

ST239-MRSA-III isolates were resistant to tetracycline and erythromycin, in line with the report of Ito *et al.* (2001), in which the *SCCmec* type III element was found to contain integrated copies of plasmid pT181, transposon Tn554 and pseudoTn554 encoding resistance to tetracycline, erythromycin and cadmium, respectively. It may have been more appropriate to determine the MIC of a wider range of antimicrobial agents to enable correlation of the degree of antimicrobial resistance with lineage as determined by MLST and *SCCmec* typing.

This is one of the first reports of the prevalent genotypes of MRSA circulating in both hospital and community settings in Pakistan. This study shows that the prevalent MRSA clones associated with hospital- and community-onset infection in Karachi, Pakistan, are ST239-MRSA-III and ST8-MRSA-IV. A close link between ST8, PFGE type B, *SCCmec* IV and CO-MRSA was found. Similarly, ST239, PFGE type A, *SCCmec* III and HO-MRSA were predominantly grouped together. However, it should be noted that only a limited number of isolates were investigated during this study, which covered a limited geographical area over a fairly short period of time. Nevertheless, the information obtained is important as it enables us to form a basis against which to monitor the future spread and emergence of strains in this region. In addition, previously undescribed variants of the *SCCmec* element have been identified. Future investigation of these elements may reveal particular characteristics that enable these strains to succeed in this setting.

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